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Original Article

Screening of potential small volume resuscitation products using a severe hemorrhage sedated swine model

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Abstract: Background: Small volumes of resuscitation products to sustain survival until definitive care are desired in extreme environments due to limited resources. A severe controlled hemorrhage model in sedated, sexually mature miniature swine has been developed to evaluate these products. Valproic Acid (VPA) and Pentoxifylline (PTX) have been suggested as potential products for small volume resuscitation following hemorrhage predominately in anesthetized small animal models. We evaluated the survival time of VPA and PTX in the swine model. Methods: Fifteen male miniature swine weighing 41.1 ± 2.9 kg were sedated and hemorrhaged 60% of estimated blood volume over 1 hr and treated with one of the following: 1) VPA at 400 mg/kg in a volume of 1.33 ml/kg over 2 min (n=4); 2) VPA at 300 mg/kg in a volume of 2 ml/kg over 30 min (n=3); 3) PTX at 50 mg/kg in a volume of 2 ml/kg over 2 min (n=4); 4) saline vehicle at 2 ml/kg over 2 min (n=4). Survival times were compared to non-resuscitated historic controls (n=16). Survival was determined from the end of hemorrhage/initiation of treatment. Results: Median (95% CI) survival times were: Control 55.7 (17.5 – 86) min; VPA (400 mg/kg) 6 (4 – 8) min; VPA (300 mg/kg) 17.5 (12 – 24.5) min; PTX 60.8 (21 – 75) min; and vehicle 92 (15 – 180) min. No treatment increased survival time compared to controls and there were no significant differences in percent survival among groups. Conclusion: In this sedated severe hemorrhage model VPA and PTX were unacceptable as small volume resuscitation products at the concentrations and delivery rates used because of early deaths. Considering that these drugs are FDA approved for other indications at lower doses the present data suggest that further investigation of mechanisms involved are warranted.

Keywords: Valproic acid (VPA); Pentoxifylline (PTX); 60% hemorrhage; metabolic acidosis; survival; mature male miniature swine

Introduction

Medical support personnel responding to multiple traumas on the battlefield or in remote locations may not have sufficient fluids for adequate resuscitation, and would therefore benefit from a small volume resuscitation product that could be given after hemorrhage control to sustain the wounded for extended time before evacuation to a medical support facility. Valproic Acid (VPA) and Pentoxifylline (PTX) have been suggested as potential small volume resuscitation products.

Valproic Acid has been used extensively at doses of 600 – 1200 mg/day, p.o. (serum level of 50 – 100 μg/ml) as an anticonvulsant, and treatment for bipolar disorders, anxiety, psychoses, alcoholism and dementia[1]. More recently, the histone deacetylase inhibitor (HDACI) activity of VPA has been demonstrated to provide hyperacetylation of histones for increased gene transcription, resulting in improved cellular protection (300 mg/kg) [2-4], hypoxia protection following lethal hemorrhage (166 mg/ml) [5], as well as increased hemorrhage survival in rats (300 mg/kg)[6] and swine (400 mg/kg)[7].

Pentoxifylline is a methylxanthine derivative, phosphodiesterase inhibitor, anti-inflammatory compound [8,9]. PTX is used clinically at maximum doses of 1200 mg/day, p.o. or i.v. for intermittent claudication and to improve cerebrovascular blood flow. In anesthetized rats (50 mg/kg) it improved survival following hemorrhagic shock [10] and intestinal blood flow (49 mg/kg) [11]. In anesthetized dogs PTX (15 mg/kg) has shown both improvements in cardiac performance and oxygen utilization [12] and systemic and regional perfusion [13], or no improvement in cardiovascular parameters [14].

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Also, using unanesthetized rats, at a PTX dose of 50 mg/kg, several studies have shown improvement in cellular function [15], cardiac output and function and tissue perfusion [16,17]. The mechanisms of PTX hemorrhage resuscitation benefits have been investigated in anesthetized rats (25 mg/kg) in hypertonic saline (HS) [18-21] or LR [22,23]. However, in an anesthetized swine study, PTX (40 mg/kg) given with 3.5% Haemaccel 1 hr after hemorrhage and sepsis, failed to attenuate organ dysfunction or improve survival [24].

VPA (400 and 300 mg/kg) and PTX (50 mg/kg) were evaluated in this investigation as small volume (1.33 or 2.0 ml/kg) resuscitation products given immediately after hemorrhage without additional fluids, using a sedated, sexually mature male miniature swine severe hemorrhage model. The model was previously developed specifically for evaluating low volume resuscitation products, as part of the Defense Advanced Research Projects Agency (DARPA) Surviving Blood Loss program [25].

Methods and materials

This investigation and the previous model development control protocol[25] were approved by the Institutional Animal Care and Use Committee of the U.S. Army Institute of Surgical Research, Fort Sam Houston, TX. The experiments were conducted in compliance with the Animal Welfare Act and Animal Welfare Regulations. All animals received care in strict compliance with the 1996 *Guide for the Care and Use of Laboratory Animals* by the National Research Council and were maintained in an Association for Assessment and Accreditation of Laboratory Animal Care International accredited facility.

Fifteen healthy, sexually mature, intact male Sinclair miniature swine weighing 41 ± 2.9 kg (mean ± SEM) were obtained from Sinclair Research Center, Inc., Columbia, MO. Health of the animals was determined with a physical exam by a veterinarian, a lung CT scan and a blood sample for CBC/blood chemistry. The animals were socialized to human activity, transport cages, laboratory procedures and trained for 2 weeks to lie quietly in a sling.

Experimental preparation

The experimental preparation has been described in detail previously [25]. Briefly: following 0.05 mg/kg of Buprenorphine, 4-5 mg/kg of Telazol, and isoflurane anesthesia the animals were catheterized in a small branch of the right carotid artery with a Data Sciences International (DSI, St. Paul, MN) telemetry transducer for arterial blood pressure; the right external jugular vein for the continuous infusion of midazolam; the right femoral artery for hemorrhage and blood samples and the right femoral vein for blood samples and infusion of the drugs. The catheters were tunneled subcutaneously to the dorsum over the shoulders and exteriorized. The incisions were closed with staples and infiltrated with bupivacaine. Isoflurane was discontinued and the animals were placed in a sling with feet on the floor and allowed to recover from anesthesia. Limb ECG electrodes were attached and BIS electrodes (Bispectral Index; Aspect Medical Systems, Newton, MA) were placed across the forehead. Midazolam infusion was started at 1.25 mg/kg/hr and adjusted throughout the study to maintain a BIS sedation level of 80-90 [26]. The animals were warmed with a heating pad and blankets to maintain a physiologic core temperature. After 30 min of stabilization, baseline hemodynamic data (systolic, diastolic and mean blood pressure and heart rate) were collected and baseline arterial and venous blood samples were taken for the following parameters: pO_2, sO_2, pCO_2, HCO_3, base excess (BE), pH, Hct, Hb, glucose, lactate, differential WBC and platelets, using standard blood gas and CBC clinical chemistry techniques. The combined volume of arterial and venous blood taken for analysis was 26 ml per sample. The animals were then hemorrhaged 60% of their estimated blood volume (65 ml/kg) exponentially over 1 hr using a computer controlled withdrawal system [25,27].

Drug administration

Immediately following end of hemorrhage (EOH), arterial and venous blood samples were collected and small volume resuscitation was started 2-3 min after EOH with one of four treatments: 1) 400 mg/kg VPA (Calbiochem, San Diego, CA, catalog # 676380, lot # D00075250) dissolved in deionized water, diluted to 1.33 ml/kg with saline and given over 2 min (4 swine); 2) 300 mg/kg VPA (lot # D00060942) dissolved in deionized water, diluted to 2.0 ml/kg with saline and given over 30 min (3 swine); 3) 50 mg/kg PTX (Calbiochem, San Diego, CA, catalog # 516354, lot # D00092360) dissolved in normal saline, diluted

to 2.0 ml/kg with saline and given over 2 min (4 swine); 4) Normal saline vehicle at 2.0 ml/kg given over 2 min (4 swine).

Doses of VPA and PTX were chosen from prior literature regarding the use of these compounds for resuscitation after hemorrhage.

Osmolarity and pH of the VPA and PTX solutions were: VPA; pH = 8.438; mOsm/L = > 1153. PTX; pH = 4.804; mOsm/L = 393.

The animals were observed and sampled after hemorrhage at 15, 30, 60, 90, 120, 150, 180, 240, 300 and 360 min or until the animal expired. Hemodynamic data were sampled continuously. Death was defined as respiratory arrest. Any animal that survived beyond 6 hrs was euthanized with 10 ml of Fatal Plus (Vortech Pharmaceuticals, Dearborn, MI), i.v.

VPA and PTX measurement:

VPA in plasma was measured after extraction, conjugation to the fluorescent probe, 7-diethylaminocoumarin-3-caroxylic acid hydrazide (DCCH, Invitrogen Corp, Carlsbad, CA), separation by reversed phase HPLC and measured by fluorometry. Samples were run in triplicate and values calculated against a standard curve generated from untreated pig plasma spiked with known amounts of VPA. 2-phenyl butyric acid was used as an internal standard.

PTX levels in plasma were determined by UV spectroscopy following extraction and HPLC separation of PTX from its metabolites as described by Sripalakit [28]. A standard curve was generated by adding known amounts of PTX to untreated pig plasma. Chloramphenicol was used as internal standard.

Statistical analysis

The primary endpoint of these experiments was an improvement in survival time compared to non-resuscitated controls with a goal to reach 3 hr based on DARPA requirements.[25] VPA, PTX or vehicle data were compared to a previously obtained untreated severe hemorrhage (60%) control model treated identically to the present animals (n = 16) [25]. Historical controls were used to reduce animal use. We have previously demonstrated a median survival time of 56 min with an IQR of 27 – 94 min in this model, which was highly reproducible over a 21 month period. The study was designed for termination of a treatment arm for futility to demonstrate an improvement, if survival time compared to control (no treatment) did not improve. This interim analysis was scheduled at n=3 or 4 in a group with an intent to include n=8 per group. As there were so few survivors beyond the end of hemorrhage, the hemodynamic and metabolic data figure is presented as an appendix so as not to distract from the survival benefit focus of the study.

All data are presented as mean ± SEM or median (95% CI). A within groups repeated measures analysis of variance was used to examine the difference between the control group and the VPA, PTX or vehicle resuscitation groups, including change over time and the interaction of time and group. The hemorrhage and the resuscitation/recovery periods were considered separately in the analysis. If the repeated ANOVA showed a significant difference between groups over time, a post hoc analysis was performed to determine which time points were significantly different using an independent *t*-test between the groups (or non-parametric equivalent, as necessary). A Kaplan-Meier estimate of survival was constructed to compare the Control vs. each other group (VPA, PTX or. the saline vehicle groups) via the stratified log rank test. A $p \leq 0.05$ was considered statistically significant.

Results

Survival

Swine weights, age, hemorrhage volume, resuscitation volume and survival time after end of hemorrhage (EOH) for the 31 swine used in these comparisons are presented in **Table 1**. All 31 animals were treated similarly from baseline through EOH, including the same hemorrhage volume. Median (95% CI) survival time from EOH for the VPA animals that received 400 mg/kg or 300 mg/kg was 6 (4 – 8) min and 17.5 (12 – 24.5) min, respectively. Animals treated with PTX had a median survival of 60.8 (21 – 75) min after EOH, whereas the saline vehicle animals had a median survival of 92 (15 – 180) min after EOH, with one of the four animals surviving to 180 min. For comparison, median survival time from EOH for the sixteen control swine (no resuscitation) was 55.7 (17.5 – 86)

Table 1. Swine Weight, Age, Hemorrhage Volume, Resuscitation Volume and Survival Time

	Weight (kg)	Age (months)	Hem. Vol. (ml)	Hem. Vol. (ml/kg)	Resuscitation (ml/kg)	Mean Survival (min)	Survival at 180 min
Control (n = 16)*	40.4 ± 1.4	13.3 ± 0.5	1565± 54	38.8 ± 0.1	none	64 ± 11.5	1/16 (6%)
VPA 400 mg/kg (n = 4)	42 ± 0.2	12 ± 0.6	1632 ± 9	38.9 ± 0.09	1.0 ± 0.2	6 ± 0.8#	
VPA 300 mg/kg (n = 3)	42.4 ± 2.6	12 ± 1	1653 ± 97	38.9 ± 0.06	1.0 ± 0.3	18 ± 3.6#	
PTX 50 mg/kg (n = 4)	40.4 ± 1.5	10.6 ± 0.9	1576 ± 58	39 ± 0	2	54 ± 12.4	
Saline Vehicle (n = 4)	39.8 ± 1.7	10.4 ± 0.7#	1547 ± 63	38.9 ± 0.16	2	95 ± 36.3#	1/4 (25%)

Data are mean ± SEM. * = Control data from Burns et al.[23] VPA = Valproic Acid; PTX = Pentoxifylline; # = significantly different than control ($p<0.05$). Percent shed blood volume replacement with VPA at 400 mg/kg and 300 mg/kg (1.0 ml/kg; corrected for early death) = 2.6%, whereas shed blood replacement with PTX or saline at 2 ml/kg was 5.1% of shed blood. Mean survival time was measured from EOH.

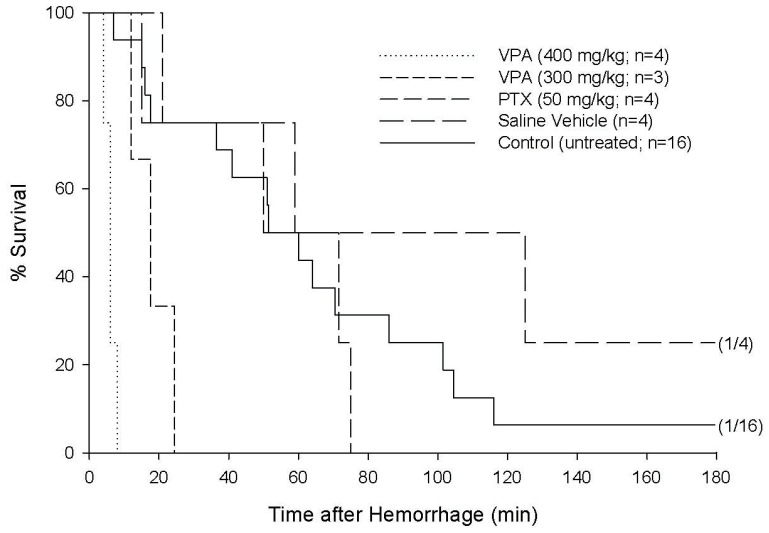

Figure 1. Kaplan-Meier survival plot of Control vs. Valproic Acid (VPA; 400 mg/kg) vs. VPA (300 mg/kg) vs. Pentoxifylline (PTX) vs. Vehicle from EOH through 180 min after hemorrhage. Number in ( ) = number of survivors at 180 min for control and vehicle.

min with a 6% (1/16) survival at the target time of 180 min. The median survival time for both VPA dosages was significantly shorter than control and vehicle (**Table 1** and **Figure 1**).

Systolic blood pressure (SBP), heart rate (HR), shock index (SI) and brain activity (BIS) data at baseline and EOH are presented in **Figure 2** for control, VPA 400 mg/kg, VPA 300 mg/kg, PTX and vehicle. Although all 31 animals were treated similarly from baseline through EOH there were significant differences in SBP between some of the groups at baseline and EOH (possibly seasonal or brood variability). All animals demonstrated a characteristic metabolic acidosis during and following hemorrhage with a significant decrease in HCO_3 (32.2 ± 0.4 to 21.8 ± 0.6 mmol/l) and base excess (BE; 7.1 ± 0.3 to -1.3 ± 0.5 mmol/l) and an increase in lactate (0.9 ± 0.07 mmol/l to 7.8 ± 0.5 mmol/l) (**Figure 2**). Core temperature of all animals averaged 38.1 ºC at baseline (n = 31), 37.9 ºC at EOH (n = 31) and 37.5 ºC at 60 min after EOH (n = 13).

All four of the swine treated with 400 mg/kg VPA died from cardiac and respiratory arrest within 4 min of start of treatment (treatment was delayed 2-3 min for blood sampling after EOH). The investigators of previous studies [2-7] were consulted and the treatment was decreased to 300 mg/kg and the administration time was increased to 30 min. All three of the 300 mg/kg VPA animals died with respiratory and cardiac arrest before the end of the 30 min infusion.

Discussion

The primary aim of the present study was to demonstrate the ability of VPA or PTX in small volumes to extend survival time compared to no resuscitation as originally defined in the DARPA Surviving Blood Loss program. The current resuscitation study, using small volumes of either VPA (1.33 or 2.0 ml/kg) or PTX (2.0 ml/kg), with

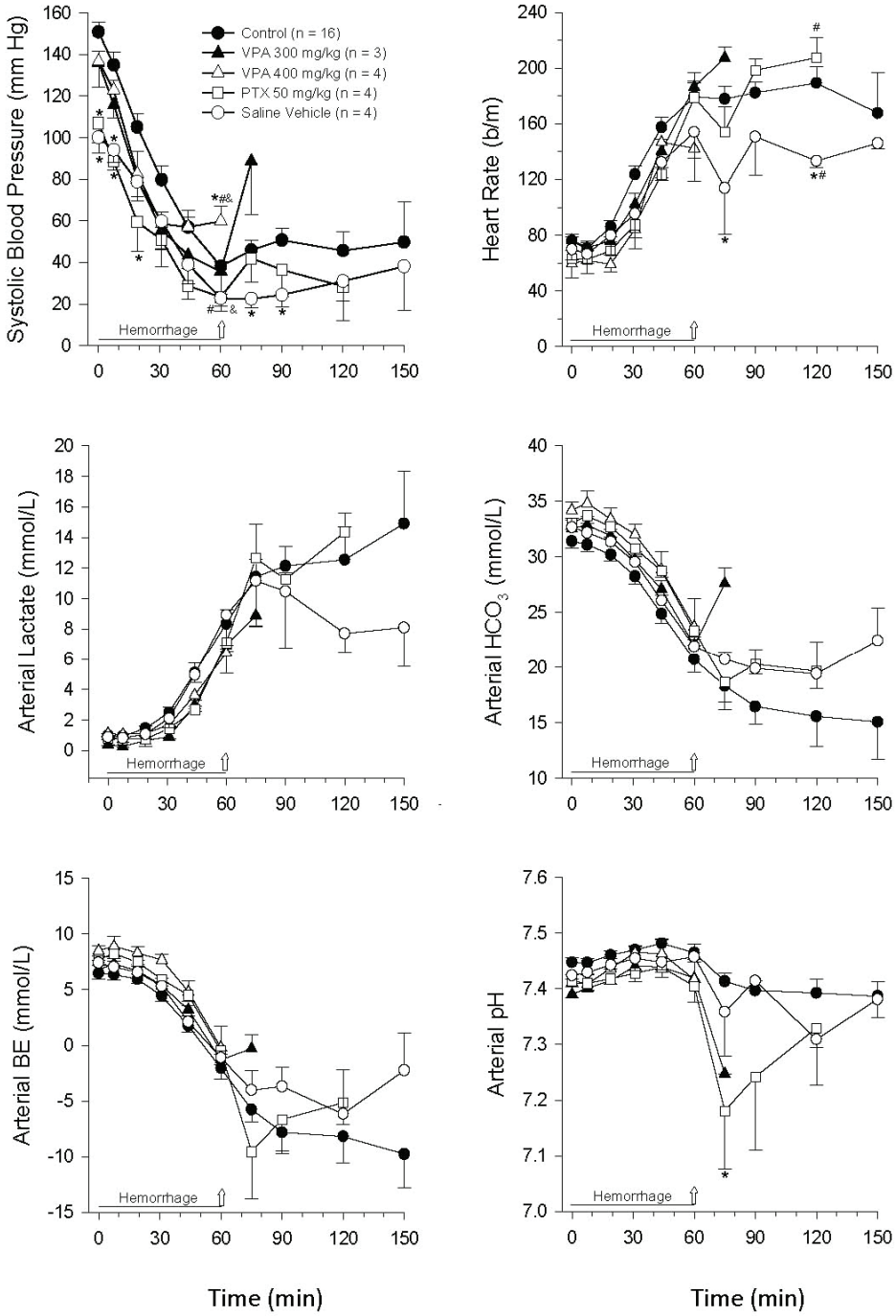

Figure 2. Hemodynamic and metabolic data. Data are mean ± SEM. Arrow indicates time of infusion of VPA, PTX or Vehicle. * = significantly different than control ($p < 0.05$). #,& = same symbol significant difference.

no additional resuscitation fluid showed dramatic negative results in the sedated, severely hemorrhaged mature male miniature swine. The response to VPA at both the 400 mg/kg and 300 mg/kg in this study appeared to be toxic with respiratory and cardiac arrest occurring rapidly after infusion. In contrast, previous studies using VPA in anesthetized rats (300 mg/kg

in 0.25 ml/kg normal saline) [2-4,6] and anesthetized swine (400 mg/kg in 30 ml normal saline plus normal saline at 3 x shed blood volume)[7] or PTX in unanesthetized rats (50 mg/kg in 2.0 ml normal saline) [15-17] and anesthetized rats (49-50 mg/kg in 42-63 ml/kg LR) [10,11], demonstrated improvements in numerous measured functions with the administration of similar doses of VPA or PTX used in the current study following hemorrhage. The disparity between the negative results from this study and the beneficial results from previous studies using the same products and similar dosages are difficult to explain.

This study, using the sedated, sexually mature male miniature swine was designed to closely simulate the mature conscious male battlefield casualty experiencing severe hemorrhage. Sedation in the present experiments was an ethical consideration. There are five significant differences between this study and previous studies using VPA and PTX in rats and swine: 1) species difference (rat vs. swine), 2) swine strain differences (Sinclair miniature swine vs. domestic swine), 3) sedation vs. anesthesia, 4) sexually mature males compared to sexually immature swine and 5) no additional fluids vs. resuscitation fluids (shed blood, hypertonic saline (HS), LR and normal saline).

The miniature swine is becoming a model of choice for hemorrhage research because of its many similarities to man [29-31]. On the other hand, the rat, with its higher metabolic rate has a different response to hemorrhage and resuscitation than the swine [25]. Anesthesia masks many of the potentially negative responses to hemorrhage and resuscitation. For example, in the current study during the infusion of PTX following hemorrhage the relaxed, sedated animals became restless, as though uncomfortable (CNS adverse overdose reaction seen in humans), with body movements such as extension of the limbs. One animal had pronounced leg extension and arching of the back with the head raised (appeared to be a seizure) and a transient HR elevation to 237 b/min from a HR of 157 b/min at the beginning of infusion. The animal's BIS sedation level was 81 and MAP did not change. These dramatic symptoms would not have been observed if the animals were anesthetized. Probably the most significant factor between this study and most previous VPA and PTX studies was the lack of supplemental fluids during resuscitation in this study.

VPA

Several recent studies involving anesthetized rats hemorrhaged 60% of EBV followed by the administration of 300 mg/kg of VPA over 2 min [6], and anesthetized swine with femur fracture, 60% hemorrhage and liver laceration followed by 400 mg/kg of VPA given over 30 min [7], demonstrated improvements in survival without fluid resuscitation in the rat study [6] or saline infusion but no shed blood transfusion in the swine study [7]. These two studies were similar, but not identical, to the current study. In the rat study [6], the animals received VPA (300 mg/kg) in 0.25 ml saline over a 2 min period after hemorrhage, plus the normal saline standard of care for 60 min and no other fluids. In the swine study [7], the VPA (400 mg/kg) was administered in 30 ml of normal saline over a 30 min period after hemorrhage, and after the administration of normal saline at 3 times the shed blood volume. It is still questionable whether these differences would account for the dramatic results noted in the present experiments.

VPA is a drug that has been accidentally or intentionally overdosed many times [32,33]. The serum therapeutic range for VPA in humans is 50-150 μg/ml [32] and the toxic and potentially fatal serum level is > 850 μg/ml [33]. During normal conditions VPA is 90% protein bound, however, at high levels of VPA (greater that 30-50 μg/ml), protein binding is saturated and a large proportion of the VPA is free, exacerbating its effects. Normal half life is 7-15 hrs [34] but can be extended by overdose.

Serum levels of VPA after a single dose of 400 mg/kg in traumatized and hemorrhaged anesthetized swine were reported as 900 μg/ml, 560 μg/ml and 400 μg/ml immediately, at 2 hr and at 5 hr after treatment, respectively [7]. Previous human serum or plasma concentrations of VPA in patients who died were 1970 μg/ml [35], 1700 μg/ml [33], 1361 μg/ml [33], 1914 μg/ml [34] and 2722 μg/ml [36]. The human deaths were not as immediate as the current swine study. However, the humans were not challenged with a 40% residual blood volume and they were being vigorously treated for the overdose. Measured plasma levels of VPA at 15 min post treatment from two of the swine treated with 300 mg/kg VPA in this study were:

1010 μg/ml and 530 μg/ml, similar to a previous report [7] and less than those associated with lethal overdose. Some of the symptoms of severe VPA overdosing reported in humans are coma, respiratory depression or arrest, cardiac arrest and acidosis [32-34]; symptoms observed in the present experiments.

PTX

PTX experiences rapid absorption, distribution, metabolism and excretion through the kidney. A single 400 mg oral dose resulted in a plasma concentration of 1.1μg/ml at 1.05 hr. There is no significant protein binding and the normal metabolic half-life is 0.89 hr.[8,9] The human therapeutic blood level of PTX is approximately 0.3-1.3 μg/ml [8,9,37]. A blood level associated with lethality in a human was 32.5 μg/ml.[37] In the current study, measured plasma levels (μg/ml) from the four PTX treated swine was 144 ± 17.4, 88 ± 10.4 and 56 ±1.0 at 15 min, 30 min and 60 min after treatment, respectively, which were higher than the level observed in a patient who died. Plasma levels in other animal studies after iv infusion of PTX were not reported, so it remains unclear whether sensitivity of humans to PTX is greater than in animals.

The use of 50 mg/kg PTX after resuscitation with lactated Ringer's (LR) at four times shed blood volume in unanesthetized rats following trauma-hemorrhage demonstrated improved liver function [15], cardiac output and tissue perfusion [16] and cardiac performance [17]. Other studies showed beneficial effects in anesthetized rats after hemorrhage [10,11,18-23]. Anesthetized dogs that were hemorrhaged to a MAP of 40 mm Hg for 30 min showed improved cardiac performance and oxygen utilization after pulmonary artery infusion of 15 mg/kg PTX with LR at two times shed blood volume plus 100 ml of LR over 45 min [12], or improvement in systemic and regional perfusion after 15 mg/kg PTX in 4 ml/kg HS [13]. Thus, resuscitation with VPA or PTX may require additional fluids such as LR, HS, normal saline or return of shed blood to eliminate the toxic effects observed here.

The number of animals in this VPA and PTX study was abbreviated because of early fatalities as pre-designated in the experimental design. Continuing with additional animals would not be statistically or ethically justified. Based on the current results, to detect a significant 180 min survival difference (0.8 power) between control (n = 16) vs. VPA (400 mg/kg; n = 4), VPA (300 mg/kg; n = 3) and PTX (n = 4) would require 122 animals in each group.

Summary

The use of VPA and PTX as small volume resuscitation products in a sedated, severe hemorrhage swine model failed to demonstrate an improvement in survival time at the doses and delivery rate used in this study in the absence of addition fluid resuscitation. Previous studies suggest that VPA and PTX may be useful as adjuncts with HS, LR or colloids for hemorrhage resuscitation. Use of sedated animals is suggested as this may elucidate adverse effects of these compounds masked by anesthesia.

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Conflict of Interest Statement

None of the authors have any conflicts of interest regarding the contents of this manuscript or the compounds that were examined.

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